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Fc receptor regulation of protective immunity against *Chlamydia trachomatis*

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SUMMARY

The prevailing paradigm for designing potentially efficacious vaccines against the obligate intracellular bacterium, *Chlamydia trachomatis*, advocates regimens capable of inducing a mucosal antigen-specific T helper type 1 (Th1) response. However, recent reports indicate that rapid and efficient clearance of a secondary infection also requires certain B-cell functions. We investigated the hypothesis that Fc receptor (FcR)-mediated antibody effector mechanisms are important B-cell-related functions involved in controlling a chlamydial genital reinfection. Microbiological analysis of genital chlamydial infection in FcR knockout (FcRKO) mice lacking the activatory FcγRI (CD64) and FcRγIII (CD16), as well as the inhibitory FcγRIIB1 (CD32), revealed a greater intensity of secondary infection (i.e. bacterial shedding) in $FcR^{-/-}$ as compared to $FcR^{+/+}$ mice; however, the course of the primary infection was indistinguishable in both animals. Pathologically, FcRKO mice suffered greater ascending infection than immunocompetent wild-type (WT) mice after a secondary infection. Immunological evaluation indicated that the presence of specific anti-chlamydial antibodies enhanced chlamydial antigen presentation for induction of a Th1 response by $FcR^{+/+}$, but not $FcR^{-/-}$, antigen-presenting cells. In addition, specific anti-chlamydial antibodies augmented both macrophage killing of infected epithelial cells by antibody-dependent cellular cytotoxicity (ADCC) and macrophage inhibition of productive growth of chlamydiae in co-cultures. These results indicate that B cells participate in anti-chlamydial immunity via FcR-mediated effector functions of antibodies, which are operative during reinfections. Such effector functions include ADCC, and possibly enhanced uptake, processing and presentation of chlamydial antigens for rapid induction of a Th1 response, all facilitating the early clearance of an infection. These findings suggest that a future anti-chlamydial vaccine should elicit both humoral and T-cell-mediated immune responses for optimal memory response and vaccine efficacy.

INTRODUCTION

Chlamydial genital infections are common, and defining the elements of protective immunity is important in the present research efforts that are being conducted with the aim of developing an efficacious vaccine for controlling the infection complications. Numerous studies in experimental animal models and corroborative reports in humans have indicated that T-cell-mediated immune responses involving the induction of $CD4^+$ T helper 1 (Th1) cells are crucial for protective immunity against *Chlamydia*.[1–5] Thus, the new paradigm for vaccine-design strategies requires regimens capable of inducing a mucosal Th1 response.[3] However, recent studies have suggested that humoral immunity is also involved in chlamydial control. Evidence supporting the contribution of anti-chlamydial antibodies to protective immunity includes the following:

(1) The association of serovar-specific antibodies in local secretions with protective immunity after *C. trachomatis* infections of humans and subhuman primates.[6–10]

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Abbreviations: DC, dendritic cells; FcR, Fc receptor; $FcR^{-/-}$, FcRKO phenotype; $FcR^{+/+}$, wild-type phenotype; FcRKO, Fc receptor knockout; PID, pelvic inflammatory disease; STD, sexually transmitted disease.

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(2) Serovar- and serogroup-specific monoclonal antibodies (mAbs) generated against the major outer membrane protein (MOMP) were able to neutralize chlamydial infectivity in tissue culture *in vitro,* protect mice from chlamydial toxicity when passively administered and prevent infection of monkey conjunctivae.[11,12]
(3) The presence of local (cervical) but not systemic (serum) anti-chlamydial immunoglobulin (Ig)A correlated with decreased shedding of chlamydiae in women with uncomplicated *C. trachomatis* cervical infection.[13]
(4) Previous studies in the guinea-pig and mouse models of chlamydial genital infection demonstrate that antibodies contribute to protective immunity.[14–19] Furthermore, a recent study revealed that although there was no difference in the course of a primary genital chlamydial infection in B-cell-deficient and control mice, the former suffered more intense disease upon reinfection than the control mice.[19]
(5) B-cell-deficient mice depleted of $CD4^+$ T cells by antibody treatment were incapable of controlling a secondary genital chlamydial infection.[20]

In general, the effector mechanisms of antibodies in antimicrobial immunity are mediated directly by the binding and neutralization of infectious agents, and indirectly via Fc receptors (FcRs), as in antibody-dependent cellular cytotoxicity (ADCC). The FcRs comprise a family of leucocyte surface molecules that bind the Fc portion of immunoglobulin, are members of the immunoglobulin gene superfamily of proteins and control important effector functions of antibodies in protective or pathological immune responses. Thus, FcRs function as important vehicles for antibodies to enhance the phagocytosis of microbes and their components or products, to foster intracellular degradation and removal from the body, and antigen processing/presentation for immune elicitation. The binding of FcRs by antigen–antibody complexes can activate immune effector cells – such as phagocytes, macrophages and polymorphonuclear cells/leucocytes (PMNs), natural killer (NK) cells, eosinophils and mast cells – bearing the receptors. Thus, macrophages and PMNs display increased phagocytosis and intracellular destruction of ingested pathogens, and NK cells, eosinophils and mast cells are triggered to secrete stored mediators with antimicrobial or physiological effects. More so, the augmented antigen uptake and processing exhibited by crucial FcR-bearing antigen-presenting cells (APCs) (such as dendritic cells and macrophages) in the presence of specific anti-chlamydial antibodies during a reinfection, may enhance Th1 activation for rapid control and clearance of *Chlamydia*. Of the different potential effector mechanisms of antibodies against pathogens, only *in vitro* neutralizing ability by antibodies has been demonstrated against *Chlamydia.*[11,12] As the *in vitro* neutralizing capacity of antibodies may not correlate with *in vivo* protective function, FcR-dependent effector functions may represent the major effector mechanism by which antibodies participate in controlling *Chlamydia* during a reinfection. To date, no studies have examined the effect of FcR-mediated effector functions of antibodies against *Chlamydia in vivo* or *in vitro*, so their effectiveness against *Chlamydia* is unknown. It is hypothesized that because *Chlamydia* is essentially an intracellular pathogen during its development, FcR-mediated effector functions of antibodies have a major role in chlamydial control during a reinfection. In the present study, specific studies employed genetically engineered FcR knockout (FcRKO) mice to evaluate the role of FcR in chlamydial clearance and anti-chlamydial immunity *in vivo.* Other studies analysed the effectiveness of FcR-mediated immune effector processes against *Chlamydia in vitro,* by assessing the ability of macrophages to kill infected epithelial cells via ADCC and restrict the productive growth of chlamydiae in the presence of anti-chlamydial antibodies. The results from these studies provide a mechanistic basis for the involvement of antibodies in controlling *Chlamydia* and furnish a better understanding of the role of humoral immune responses in anti-chlamydial immunity.

MATERIALS AND METHODS

Chlamydia *stocks and antigens*

Stocks of the *C. trachomatis* agent of mouse pneumonitis (*Chlamydia muridarum* or MoPn) infections were prepared by propagating elementary bodies (EBs) in McCoy cells, as described previously.[21] Stocks were titrated by infecting McCoy cells with different dilutions of EBs, and the infectious titre was expressed as inclusion-forming units per millilitre (IFU/ml). Chlamydial antigen was prepared by growing MoPn in HeLa cells and purifying EBs over renografin gradients, followed by inactivation under ultraviolet (UV) light for 3 hr.

Animals, infection and analysis of the course of the infection

Female $FcR^{-/-}$ on (C57BL/6:129) background, lacking the activatory FcγRI (CD64) and FcRγIII (CD16), as well as the inhibitory FcγRIIB1 (CD32), were developed by gene-targeting inactivation in the laboratory of Dr Jeffrey Ravetch at The Memorial Sloan Kettering Cancer Institute (University of California at San Francisco, CA). The animals and control $FcR^{+/+}$ mice were obtained from Taconic Farms (Germantown, NY) when 5–8 weeks of age. All animals received food and water *ad libitum* and were maintained in Laminar flow racks under pathogen-free conditions of 12-hr light and 12-hr darkness. Mice were infected intravaginally with 10^5 IFU of MoPn per mouse in a volume of 30 μl of phosphate-buffered saline (PBS) while under phenobarbitol anaesthesia. All mice received 2·5 mg each of Depo-Provera (medroxy-progesterone acetate; The Upjohn Co., Kalamazoo, MI) by subcutaneous route in 100 μl of PBS 7 days prior to infection, to stabilize the oestrous cycle, prevent endometrial sloughing and facilitate a productive infection manifesting chlamydial shedding, salpingitis and infertility.[22–24] The course of the infection was monitored by periodic (every 3 days) cervico-vaginal swabbing of individual animals. *Chlamydia* was isolated from the swabs in tissue culture according to standard

methods, and inclusions were visualized and enumerated by immunofluorescence.[21] The animals were monitored for 4 to 6 weeks, a time-period that spans the course of MoPn infection in mice.[1,2] Groups of animals were reinfected 85 days after the primary infection with 10^5 IFU of MoPn per mouse and either swabbed for isolation of chlamydiae from the vaginal vault and/or killed to obtain the entire reproductive system for assessment of ascending infection, as described below. Infected KO mice did not show any clinical evidence of overt pathology besides the shedding of chlamydiae in their genital tracts, suggesting that the inoculum was not lethal for the animals. Experiments were repeated to include 10 or 12 animals per experimental group.

Assessment of ascending infection. MoPn was isolated from the upper genital tracts of mice at different time-points after genital infection. In brief, mice were reinfected intravaginally with 10^5 IFU of MoPn per mouse 85 days after the primary infection, as previously described.[21] (It has been established that mice become susceptible to reinfection by this time after a primary infection.[1]) At the indicated time-points after infection, a portion of the reproductive system between the uterus and the ovaries of each mouse was removed, teased and homogenized, and tissue homogenates were collected in 1 ml of PBS. *Chlamydia* was isolated from the homogenate in tissue culture according to a standard immunofluorescence staining method.[21]

Cytokines, mAbs and other reagents

Enzyme-linked immunosorbent assay (ELISA) kits for quantifying the amounts of murine cytokines in biological and culture fluids were purchased from BioSource International (Camarillo, CA). Chlamydial isolation from cervicovaginal swabs in tissue culture was assayed by staining infected monolayers of McCoy cells with fluorescein isothiocyanate (FITC)-labelled, genus-specific anti-chlamydial antibodies (Kallestad Diagnostics, Chaska, MN) for detection of chlamydial inclusions by direct immunofluorescence.[21] High-titre mouse anti-MoPn antibodies were prepared by 50% ammonium sulphate precipitation of the gamma globulin fraction of hyperimmune sera from C57BL/6 mice infected multiple times with MoPn by the genital route, as previously described.[25] Following antibody concentration and purification by dialysis with PBS, the protein content was determined by using the standard Lowry method. The specificity and effectiveness of this anti-MoPn antibody preparation was established by staining MoPn-infected McCoy cells (at a dilution of 1 : 1000) to reveal chlamydial inclusions by immunofluorescence, according to a standard procedure.[21] High-titre mouse anti-*Mycobacterium tuberculosis* (anti-TB) serum antibodies (prepared in a similar manner) were kindly supplied by Dr Ram Navalkar (Morehouse School of Medicine, Atlanta, GA).

Preparation of peritoneal exudate macrophages (PEMs)

The procedure for preparing large numbers of PEMs from mice was as previously described.[26] Briefly, each mouse received 0·50 ml of 3% thioglycolate by the intraperitoneal route. The mice were killed after 5 days by cervical dislocation, and the peritoneal cavities were washed with 10 ml of warmed PBS per mouse. The cells were washed three times, enumerated and resuspended in complete RPMI-1640 medium.

Effect of specific anti-chlamydial antibodies on chlamydial antigen presentation for Th1 activation by splenic APCs from $FcR^{-/-}$ and $FcR^{+/+}$ mice

The efficiency of antigen presentation by splenic APCs from FcRKO and wild-type (WT) mice for specific Th1 activation in the presence of specific anti-chlamydial antibodies was compared by assessing the ability of γ-irradiated whole spleen cells to present chlamydial antigens to immune T cells from infected WT mice. Spleen cells from chlamydia-infected WT mice were enriched for T cells by the nylon wool-adherence method.[27,28] Purified splenic cells contained at least 90% $CD3^+$ cells, as determined by fluorescence-activated cell sorter (FACS) analysis. To assess the effect of specific anti-chlamydial serum on the antigen-presenting function of γ-irradiated splenic cells from either FcRKOs or control mice, 2×10^5 splenic cells were co-cultured with 2×10^5 nylon wool-purified T cells in the presence or absence of chlamydial antigen (i.e. UV-inactivated MoPn EBs at 10 μg/ml) and specific anti-chlamydial antibodies (50 μg/ml) in 96-well tissue culture plates. At the end of each incubation period, the supernatants were collected and assayed for IFN-γ content by a quantitative ELISA (Cytoscreen™ Immunoassay Kit; BioSource) according to the supplier's instructions. The concentration of the cytokine in each sample was obtained by extrapolation from a standard calibration curve generated simultaneously. Data were calculated as the mean values [$\pm$standard deviation (SD)] of triplicate cultures for each experiment. The results were derived from at least three independent experiments.

Assessment of ADCC against chlamydial-infected epithelial cells

A modified ADCC assay based on enumeration of dead cells in co-cultures of infected epithelial cells and macrophages was developed as an alternative to the conventional radioactive ^{51}Cr-release assay. The mouse epithelial cell line, TM3 (ATCC CRL 1714; American Type Culture Collection, Rockville MD), derived from BALB/c mice,[29] was maintained in complete RPMI-1640 medium. It has been established that TM3 cells efficiently support the growth of MoPn in culture.[30] TM3 cells (10^4/well) were plated in flat-bottomed 96-well plates and allowed to attach for $\approx$6 hr. The cells were infected with MoPn [at a multiplicity of infection (MOI) of 1] for 12 hr (to allow expression of MoPn antigens on the surface) and were then washed three times with sterile PBS. Mouse anti-MoPn or anti-TB antibodies were added at 50 μg/ml to appropriate wells and incubated for 15–20 min, before adding PEMs (10^5/well), as indicated. Cultures were incubated for an additional 12 hr, washed with PBS and stained with Trypan Blue to assess cell ADDC by enumerating dead (Trypan Blue

uptakers) and live (Trypan Blue excluders) cells in 10 fields of a ×40 eyepiece light microscope. The concentration of antibodies used was determined to be non-toxic for the cells in culture. Cultures were established in triplicate, and each set of experiments was repeated at least three times to obtain a quantifiable and consistent pattern of results. Percentage cytotoxicity was determined from the following formula:

$$\% \text{ Cytotoxicity} = (\text{mean of dead cells in 10 fields} \div \text{mean of total cells in 10 fields}) \times 100.$$

Assessment of FcR-mediated inhibition of chlamydiae by PEMs in co-cultures

TM3 cells (10^4/well) were plated in flat-bottomed 96-well plates and allowed to attach for ≈6 hr. The cells were infected with MoPn (MOI = 1) for 12 hr (to allow expression of MoPn antigens on the surface) and then washed three times with PBS. Mouse anti-MoPn or anti-TB antibodies were added to appropriate wells at 50 μg/ml before adding PEMs (10^5/well), as indicated. Culture incubation was extended to 48 hr, after which each well was scraped and the cells collected in tubes, sonicated briefly and used to inoculate McCoy cells for isolation of chlamydiae in tissue culture by standard immunofluorescence.[21] Cultures were established in triplicate, and the results were expressed as IFU/ml (mean ± SD). Each set of experiments was repeated at least three times to obtain a quantifiable and consistent pattern of results. The percentage inhibition was determined as follows:

$$\% \text{ Inhibition} = [(\text{mean IFU/ml of control cultures} - \text{mean IFU/ml of experimental cultures}) \div \text{mean of control cultures}] \times 100.$$

Statistical analysis

The levels of IFN-γ in samples from different experiments, and the degree of cytotoxicity or inhibition, were analysed and compared by performing a one- or a two-tailed *t*-test, and the relationship between different experimental groupings was assessed by analysis of variance (ANOVA). Minimal statistical significance was judged at $P < 0{\cdot}05$.

RESULTS

Course of genital chlamydial infection in FcRKO and WT mice

The pathological effect of FcR deficiency on the ability of female C57BL/6 mice to efficiently clear primary and secondary chlamydial genital infections was investigated by comparing the course of chlamydial genital infection in FcRKO and WT mice. Figure 1 reveals that the course of a primary genital chlamydial infection in FcRKO and WT mice was essentially identical, indicating that FcR deficiency has no discernible pathological consequence on the microbiological clearance of chlamydia from the genital tract. However, FcRKO mice exhibited a protracted and more intense secondary infection (Fig. 2). In addition, FcRKO

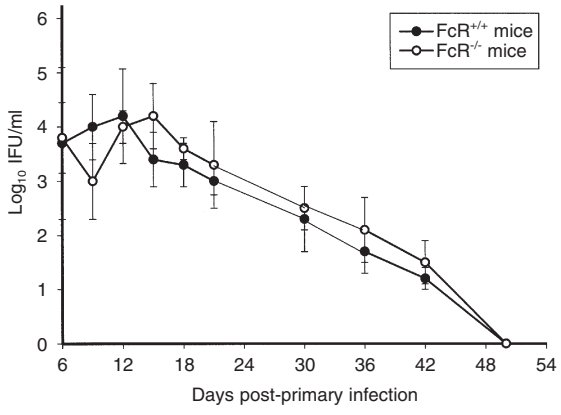

Figure 1. Course of a primary genital chlamydial infection in Fc receptor knockout ($FcR^{-/-}$) and Fc receptor wild-type ($FcR^{+/+}$) mice. Female $FcR^{-/-}$ and $FcR^{+/+}$ mice, 5–8 weeks old, were infected intravaginally with 10^5 infection-forming units (IFU) of *Chlamydia muridarum*, the *Chlamidia trachomatis* agent of mouse pneumonitis (MoPn). The course of infection was monitored by periodic (every 3 days) cervico-vaginal swabbing of individual animals. Chlamydiae were isolated from the swabs in tissue culture, according to standard methods, and inclusions were visualized and enumerated by immunofluorescence.[49] Results are expressed in IFU/ml. Experiments were repeated twice, using 10 or 12 animals per experimental group. Error bars represent the standard error of the mean (SEM).

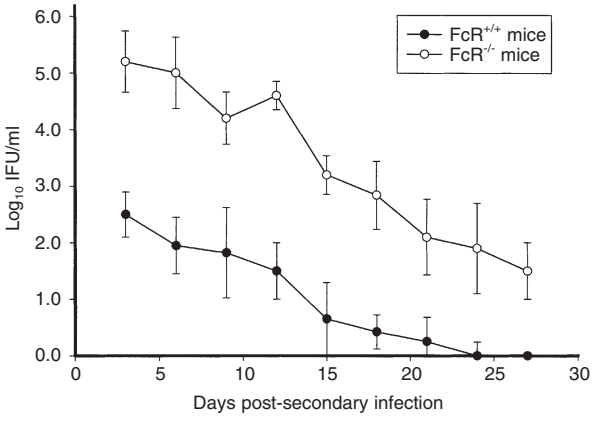

Figure 2. Course of a secondary genital chlamydial infection in Fc receptor knockout ($FcR^{-/-}$) and Fc receptor wild-type ($FcR^{+/+}$) mice. Groups of animals were reinfected 85 days after resolution of the primary infection with 10^5 infection-forming units (IFU) per mouse of *Chlamydia muridarum*, the *Chlamidia trachomatis* agent of mouse pneumonitis (MoPn). The course of infection was monitored by periodic (every 3 days) cervico-vaginal swabbing of individual animals. Chlamydiae were isolated from the swabs in tissue culture, according to standard methods, and inclusions were visualized and enumerated by immunofluorescence.[49] Results are expressed in IFU/ml. Experiments were repeated twice, using 10 or 12 animals per experimental group. Error bars represent the standard error of the mean (SEM).

mice suffered a greater ascending infection, as shown by the presence of a greater number of live chlamydiae in the upper genital tract (265·34 ± 16 FcRKO versus 12·32 ± 1·4 WT; $P > 0{\cdot}002$) on day 7 after the challenge infection. The results suggested that host defence processes of antibodies specifically involving FcRs are irrelevant for chlamydial clearance during a primary infection; however, FcR-mediated immune-effector processes of antibodies are involved in the efficient clearance of *Chlamydia* during a reinfection. Two complementary hypotheses relating to enhancement of effector function of phagocytes and potentiation of Th1 activation were therefore investigated to elucidate the molecular and cellular bases for FcR involvement in anti-chlamydial immunity.

Macrophage killing of chlamydial-infected epithelial cells by ADCC

The first hypothesis tested to study the involvement of FcR-mediated effector functions of antibodies in anti-chlamydial immunity is that antibodies can kill chlamydial-infected epithelial target cells by ADCC. As shown in Table 1, the presence of specific anti-chlamydial antibodies (anti-MoPn) increased macrophage killing of chlamydial-infected epithelial cells by approximately fourfold ($P > 0{\cdot}003$), revealing the involvement of an antibody-dependent killing mechanism. An irrelevant anti-TB antibody or normal mouse antibodies had no effect on the degree of PEM killing of infected cells. In addition, the presence of anti-chlamydial antibodies had no effect on ADCC exhibited by PEMs from FcRKO mice. These results may represent the first conclusive demonstration of the effectiveness of ADCC against *Chlamydia*. It is therefore conceivable that pre-exposed individuals harbouring specific anti-chlamydial antibodies probably control the extent of chlamydial replication by killing of the infected epithelial cells. However, it is unknown whether ADCC also leads to the killing of intracellular chlamydiae.

Table 1. Role of antibody-dependent cellular cytotoxicity (ADCC) in chlamydial control

Cultures*	% Cytotoxicity (±SEM)
TM3 cells + MoPn	8·20 (2·1)
TM3 cells + MoPn + Anti-MoPn	5·30 (0·5)
TM3 cells + MoPn + Anti-TB	4·22 (2·1)
TM3 cells + MoPn + PEMs	22·62 (3·3)
TM3 cells + MoPn + Anti-MoPn + PEMs	79·66 (2·3)
TM3 cells + MoPn + Anti-TB + PEMs	19·80 (2·6)
TM3 cells + MoPn + KO-PEMs	15·80 (5·2)
TM3 cells + MoPn + Anti-MoPn + KO-PEMs	18·60 (6·3)
TM3 cells + MoPn + Anti-TB + KO-PEMs	20·40 (4·1)

*% Cytotoxicity in control cultures containing TM3 cells + antibodies to *Mycobacterium tuberculosis* (anti-TB), TM3 cells + antibodies to *Chlamydia muridarum* (anti-MoPn; the *C. trachomatis* agent of mouse pneumonitis), TM3 cells + peritoneal exudate macrophages from wild-type mice (PEMs), TM3 cells + anti-MoPn + PEMs, and TM3 cells + anti-TB + PEMs were 0·0, 0·0, 2·10 (1·5), 3·15 (1·6), and 2·52 (1·4), respectively. Percentage cytotoxicity in equivalent control cultures containing PEM from FcRKO mice (KO-PEM) was less than 3·0%. A total of 10^4 TM3 cells were plated per well.

SEM, standard error of the mean.

Enhanced macrophage killing of chlamydiae in the presence of anti-chlamydial antibodies

We investigated the possibility that concomitant with ADCC was an enhanced inhibition of productive development of chlamydiae in epithelial cells co-cultured with PEMs in the presence of specific anti-chlamydial antibodies. The results presented in Fig. 3 show that when the level of chlamydial multiplication was assessed in co-cultures of infected epithelial cells and PEMs, there was a greater inhibition of the productive growth of chlamydiae in cultures pretreated with anti-chlamydial antibodies (anti-MoPn) prior to addition of PEMs ($P > 0{\cdot}011$). The antibody had no effect on the intracellular development of chlamydiae in the absence of PEM from wild-type mice, or on PEMs from FcRKO mice. Also, an irrelevant anti-TB antibody had no effect on the level of

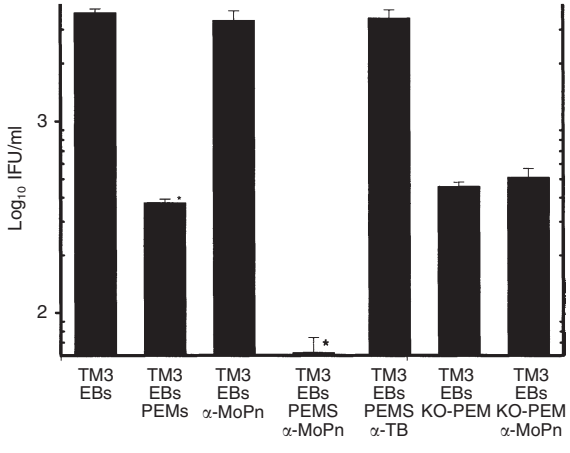

Figure 3. Enhanced macrophage killing of chlamydiae in the presence of anti-chlamydial antibodies. TM3 cells (10^4/well) were plated in flat-bottomed 96-well plates and allowed to attach for ≈ 6 hr. The cells were infected with elementary bodies (EBs) of *Chlamydia muridarum*, the *Chlamidia trachomatis* agent of mouse pneumonitis (MoPn), at a multiplicity of infection (MOI) of 1, for 12 hr to allow expression of MoPn antigens on the surface. Mouse anti-MoPn or anti-*Mycobacterium tuberculosis* (TB) antibodies were added to appropriate wells at 50 μg/ml, followed by peritoneal exudate macrophages from wild-type (PEMs) or Fc receptor knockout (KO-PEMs) mice, at 10^4/well, as indicated. Culture incubation was extended to 48 hr, after which each well was scraped and the cells collected in tubes, sonicated briefly and used to inoculate McCoy cells for identification of chlamydiae in tissue culture by using a standard immunofluorescence method.[21] Cultures were established in triplicate, and the results were expressed as infection-forming units (IFU)/ml (mean ± standard deviation). Each set of experiments was repeated at least three times in order to obtain a quantifiable and consistent pattern of results. The percentage inhibition was determined as described in the Materials and methods.

chlamydial killing by PEMs. The results suggested that the presence of anti-chlamydial antibodies enhanced the killing of chlamydial-infected cells and restricted chlamydial multiplication.

Effect of specific anti-chlamydial antibodies on chlamydial antigen presentation for specific Th1 activation by APCs from $FcR^{+/+}$ and $FcR^{-/-}$ mice

Beside the potential role of macrophages in ADCC and immune activation against *Chlamydia*, FcR-bearing dendritic cells (DC) have been recognized as the major carriers of antigen from the genital lumen to the regional lymph nodes for processing, presentation and T-cell activation against *Chlamydia*.[31–33] Therefore, a second hypothesis – that antibodies can enhance Th1 activation by increasing the uptake and presentation of chlamydial antigens – was tested. Splenic APCs from FcRKO or WT mice were used to activate chlamydial immune T cells in the presence or absence of anti-chlamydial antibodies (anti-MoPn). As shown in Fig. 4, the presence of anti-chlamydial antibodies boosted the Th1 response approximately fivefold over the antigen-stimulated cultures ($P > 0{\cdot}001$). An irrelevant anti-TB antibody or the use of FcRKO APCs had no Th1-augmenting effect. Also, anti-chlamydial antibodies do not have an effect on T cells in the absence of APCs. These results indicated that in addition to fostering ADCC and inhibition of chlamydiae by macrophages, anti-chlamydial antibodies can also enhance chlamydial antigen presentation to augment the Th1 response.

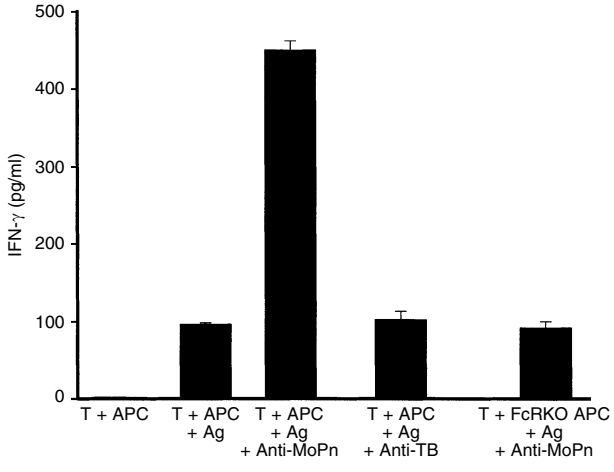

Figure 4. Effect of specific anti-chlamydial antibodies on antigen presentation for T helper 1 (Th1) activation. After 45 days of infection, spleen cells from chlamydial-infected wild type (WT) mice were enriched for T cells by using the nylon wool-adherence method.[27,28] Purified T-cell preparations (T) were stimulated with splenic antigen-presenting cells (APCs) from either Fc receptor knockout (FcR KO) or FcR WT mice plus chlamydial antigen, i.e. UV-inactivated mouse pneumonitis (MoPn) elementary bodies (EBs) at 10 μg/ml, in the presence or absence of specific anti-chlamydial antibodies (anti-MoPn) or an irrelevant antibody preparation [anti-Mycobacterium tuberculosis (anti-TB)] at 50 μg/ml, in 96-well tissue culture plates for 5 days. At the end of the incubation period, the supernatants were collected and assayed for interferon-γ (IFN-γ) content by using a quantitative sandwich enzyme-linked immunosorbent assay (ELISA), as described previously.[46] The concentration of the cytokine in each sample (pg/ml) was obtained by extrapolation from a standard calibration curve generated simultaneously. Data were calculated as the mean values (±SD) of triplicate cultures for each experiment. The results were derived from at least three independent experiments.

DISCUSSION

The challenge posed by the need to develop an efficacious anti-chlamydial vaccine to curb the severe complications of genital infections has imposed an urgent requirement to define the immune effectors relevant to immunity and to identify the antigens and delivery vehicles that would foster the induction of those effectors. Results from experimental animals and corroborative clinical studies have established a crucial role for Th1-mediated cell-mediated immunity (CMI) in anti-chlamydial immunity.[1–5] However, there is mounting evidence for an important (although ancillary) role for the humoral immune response in immunity against *Chlamydia*.[13,19,20] The mechanisms by which the humoral immune response contributes to anti-chlamydial immunity are unclear but may include antibody-mediated neutralization of infectious particles and FcR-dependent functions. The high efficiency of B-cell-deficient mice in resolving a primary genital or pulmonary chlamydial infection,[19,21,34] but the inefficient capacity to clear a secondary infection,[20,34] suggested that antibody may be more important for controlling cases of reinfection than a primary infection. Thus, the pathological consequences of antibody deficiency in immunity against *Chlamydia* have been observed during a secondary or reinfection when certain functions associated with specific antibodies are obviously absent from the immunocompromised host.[19,20,34] Indeed, by analysing FcRKO mice, we have confirmed in the present studies that FcRKO mice can control a primary genital chlamydial infection as efficiently as their immunocompetent WTs. However, FcRKO mice suffered a greater secondary infection marked by higher multiplication of chlamydiae in the genial tract, and consequently greater ascending disease.

Potentially, the role of antibodies in controlling *Chlamydia* during a secondary infection would involve three major mechanisms. The first is antibody neutralization of free infectious particles. As *Chlamydia* is an intracellular pathogen, usually restricted to the epithelial tissue, chlamydiae are exposed to antibodies for neutralization only when released from infected cells or during reinfection of pre-exposed individuals. Secretory IgG and IgA are probably involved in this process if they are present in the interstitial spaces or in mucosal secretions during chlamydial infection. The second potential effector mechanism for humoral immune control of chlamydiae is antibody augmentation of chlamydia particle ingestion and destruction by phagocytes following opsonization of free particles or infected cells by antibodies (an FcR-dependent process).

Macrophages and PMNs are probably involved in this process and these cells are induced during genital chlamydial infection. An enhanced antimicrobial action of macrophages and PMNs is therefore predicted in pre-exposed individuals with specific anti-chlamydial antibodies. In fact, macrophages have been shown to contribute to anti-chlamydial immunity[35] although it was unclear whether the mechanism was accessory as in antigen presentation or as effector cells. Also, PMNs have been shown to take up and degrade chlamydiae via the intracellular antimicrobial peroxidase system.[36] In the case of FcR-mediated uptake of chlamydiae into DC and macrophages, the outcome is probably enhanced processing and presentation of chlamydial antigens for T-cell activation.[37–39] Although the FcR-bearing macrophages and DC have been shown to be involved in anti-chlamydial immunity,[31–33,35,40–44] the present study reveals that a major mechanism for the role of these cells is enhanced antigen presentation for Th1 induction (Fig. 4). As a third potential effector mechanism of antibody against chlamydiae, the binding of specific antibodies to chlamydial antigens expressed on the surface of infected cells could signal the cells for destruction by macrophages or NK cells via FcR-dependent ADCC. ADCC represents a mechanism by which antibodies can, through engaging Fc receptors, direct antigen-specific attack by an effector cell lacking specificity for antigen. We have shown that the presence of specific anti-chlamydial antibodies supports ADCC against chlamydial-infected cells and enhanced killing of chlamydiae in infected epithelial cells, representing perhaps the first demonstration of an FcR-driven effector function against *Chlamydia*. Besides, a potential role for NK cells in anti-chlamydial immunity during reinfection is suggested by their expression of CD16 or the FcγRIII, which binds IgG for ADCC, and NK cells have been associated with Th1 development and early clearance of chlamydia in mice.[45] As the FcR deficiency evaluated in this study [i.e. FcγRI (CD64) and FcRγIII (CD16)] excludes the receptor for IgA (CD89), the role of the latter in mediating protection during a secondary infection is unclear. However, the function of CD89 is clearly insufficient to mediate protective humoral immunity during a secondary infection as the FcγRKO mice were more susceptible than WT mice to a reinfection. Alternatively, if CD89 played a role in the response of these mice to a reinfection, it is possible that the absence of CD89 could have resulted in yet greater disease. We have shown that protective immunity involving antibody measurement correlated better with the Th1-associated mucosal IgG2a than IgA,[46] and others have demonstrated that IFN-γ receptor-deficient mice could not resolve genital chlamydial infection, even in the presence of high-titre secretory IgA in the genital tract.[47] The extent to which CD89 is involved in mediating anti-chlamydial immunity is therefore uncertain.

The simultaneous enhancement of the Th1 response and facilitation of ADCC by specific anti-chlamydial antibodies may synergistically operate to bring about the rapid resolution of a secondary infection, the situation that usually occurs.[1] Predictably, FcRKO mice will have a lower frequency of a local Th1 response during a reinfection. Antimicrobial and macrophage-activating cytokines elaborated through up-regulation of the Th1 response (e.g. IFN-γ and TNF-α) may further augment the host response and chlamydial clearance. Thus, the ability to induce an adequate humoral immune response should be an important component of a potential anti-chlamydial vaccine, in order to efficiently and effectively control chlamydial infections.

Finally, there is a potential immunotherapeutic application of the phenomenon of immune potentiation by specific antibodies in vaccine design, involving the use of specific anti-chlamydial antibodies as carriers for inducing high levels of a Th1 response. A testable hypothesis is that a faster anti-chlamydial Th1 response could be established by vaccination with immune complexes of chlamydial antigens and their specific antibodies than vaccinating with chlamydial antigens alone. The selection of specific antibody isotypes that are capable of this function may be important for the success of this procedure, as the immunization protocol employed for generating the antibodies (intravaginal infection) used in these studies was biased towards inducing Th1-associated antibodies, such as IgG2a. Thus, specific antibody isotypes capable of binding to the high-affinity CD64 (FcγRI) may function as delivery vehicles for generating immunity against certain antigens. In this respect, it has been shown that Th1-associated IgG2a supports induction of a T-cell response when used to deliver specific antigens.[48] Judicious selection of an immunization route that would minimize the potential deleterious effects of immune complexes will be important before such a vaccine regimen can be extended beyond experimental models. However, these findings enhance the prospect for an immunotherapeutic regimen comprising specifically constructed antibodies against specific antigens as delivery vehicles for enhancing a primary Th1 immune response in a vaccine design strategy.

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